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TITLE: Enhanced Chronic Pain Management Utilizing
Chemokine Receptor Antagonists

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14. ABSTRACT The time from initiation of the project (15-July-2015) until the first experiments could be conducted (January, 2016) was devoted primarily to obtaining IACUC approval (October 30, 2015), the subsequent ACURO approval (December 31, 2015), hiring new personnel, conducting baseline testing for procedures not involving animals, testing equipment, developing detailed experimental design, conducting required training and testing of all personnel, and setting up all necessary accounts. Beginning in January of 2016 and through the middle of July, we conducted experiments as per the SOW. Specifically, the formalin test was standardized and the morphine dose-response completed for this as well as the rat cold water tail-flick test. Carrageenan studies were conducted and effects of the compound on swelling and pain determined, along with initial experiments on the chemokine receptor antagonist (CRA) AMD3100. Methods were established for measuring a panel of chemokines and cytokines in tissue in the formalin and carrageenan assay. In year 2, it is planned to solidify studies with the formalin assay, the cold water test, and the carrageenan assay, and to complete studies with the combinations of morphine and CRAs in these tests. In selected assays, oxycontin will be compared to morphine using the CRAs. Studies will be initiated with the incisional pain model.				
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Progress Report: July 15, 2015 to July 14, 2016

1. INTRODUCTION:

We hypothesized that chemokines (small proteins produced by cells of the immune system released from injured cells during inflammatory processes), affect the ability of opioid drugs to counteract pain. We predicted that one way of increasing the effectiveness of the pain-relieving drugs would be to eliminate or reduce the activity of the chemokines by administering chemokine receptor antagonists (CRAs). The blockade of one or more chemokine receptors would not only diminish various types of pain, but could also increase the efficacy of given doses of opioid analgesics. Thus, taking CRAs in combination with opioids will improve pain control and lowering the dose of the opioid would avoid undesirable and adverse drug effects. With more effective pain control and lower doses of opioids, treatment of conditions such as PTSD, for which drug cocktails that may include opioids are used, would also be improved. Several mouse and rat models of acute and chronic pain having varying degrees of inflammatory components are being utilized. The models are hindpaw injection of carrageenan (acute pain), cold water tail immersion, formalin (persistent pain), the hindpaw skin incision (post-operative pain), and chemically induced neuropathic pain. In order to determine if specific CRAs alter levels of selected chemokines and cytokines in the different pain models, serum and lymphoid tissues are being analyzed utilizing molecular and protein arrays, as well as multiplex quantitative analysis of protein levels of these molecules.

2. KEYWORDS:

Pain treatment; Analgesia; Nociception; Antinociception; Inflammation; Chemokines; Chemokine receptor antagonists; Opioid analgesics; Animal models of pain; Chemokine and cytokine measurements

3. OVERALL PROJECT SUMMARY:

This project involves testing the effects of chemokine receptor antagonists in 5 pain models in rats and mice. The cold water tail-flick test (CWT), performed in rats, and the formalin test, performed in mice, were already well established in our laboratories. During this first year of the project, the carrageenan test was validated in our laboratories with the new personnel. The test uses a Hargreave's instrument to measure the sensitivity to an infrared beam of rats injected into the paw with carrageenan. Paw withdrawal time is the endpoint. The apparatus also permits measurement of edema using a digital plethysmometer (Model 7150, Ugo Basile, Italy) to measure paw swelling. Dr. Cowan supervised the assembly and workings of the instrument and the training of the technical personnel in this assay. A number of preliminary tests were carried out to examine the best source of carrageenan, determine whether dorsal versus plantar injection of the irritant was optimal, and the optimal time to make the measurements post irritant injection. In addition, there were several sources from which to obtain the AMD3100. Two of these were compared using an ongoing protocol in Dr. Rawls'

laboratory, and one was chosen. All members of the team had to undergo training to handle AMD3100 because it is classified as a teratogenic substance. This training was completed.

Major task 1: Obtain IACUC/ACURO approvals and hire and train new technical personnel

During this first year of the project, the IACUC and the ACURO approvals were obtained. The final approval for the IACUC was October 30, 2015 and for the ACURO, December 31, 2015. One full-time Associate Scientist, Xiaohong Chen, was hired in August, 2015. A part-time Laboratory Manager, Joseph Meissler, also began in August, 2015. A full-time Research Assistant, Jason DiCola, was engaged in early January, 2016. A part-time laboratory technician, Mia Watson, was hired in February 2016. All personnel passed the required tests to carry out animal experiments by mid-February.

General Methods: Animals. Sprague-Dawley male rats (Taconic Biosciences, Germantown, NY) weighing 120-150 g, were housed in groups of 2 for a least 1 week in an animal room maintained at 22 \pm 1°C and approximately 50 \pm 5% relative humidity. Lighting was on a 12/12-hour light/dark cycle (lights on at 7:00 and off at 19:00). The animals were allowed free access to food and water. Swiss Webster male mice (Taconic Biosciences) were purchased at 15-20 g, and housed 5 per cage.

Specific Aim 1: Major Task 2: Subtask 1: Test the chemokine receptor antagonists (CRAs), AMD3100 and Maraviroc, each separately, in 3 pain models: Cold water tail-flick test, carrageenan, and formalin.

Cold water tail-flick test (CWT): The latency to flick the tail in cold water was used as the antinociceptive index, according to a standard procedure in our laboratories. A 1:1 mix of ethylene glycol:water was maintained at –3°C with a circulating water bath (Model 9500, Fisher Scientific; Pittsburgh, PA). Rats were held over the bath with their tails submerged approximately halfway into the solution. All animals were tested at 60, 15 and 0 min before drug injection. For each animal, the first reading was discarded and the mean of the second and third readings was taken as the baseline value. Latencies to tail flick after injection of drug(s) were expressed as percentage change from baseline. The percent of maximal possible antinociception (MPA%) for each animal at each time was calculated using the following formula: $\%MPA = [(test\ latency - baseline\ latency)/(60 - baseline\ latency)] \times 100$. A cutoff limit of 60 seconds was set to avoid damage to the tail.

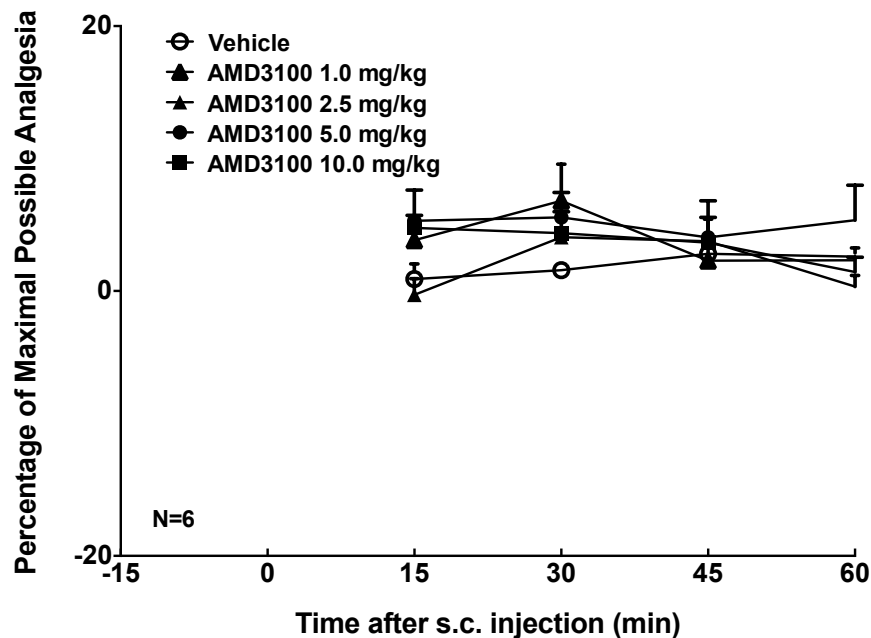


Figure 1. Antinociceptive effect of AMD3100 in the CWT. Rats (n=6/group) were injected s.c. into the dorsal surface of the body with vehicle (water) or AMD3100 at time=0 min. Each animal was tested at 15, 30, 45 and 60 min after AMD3100 injection in the CWT. AMD3100 produced no significant antinociceptive effect ($p>0.05$, ANOVA test) in the CWT test.

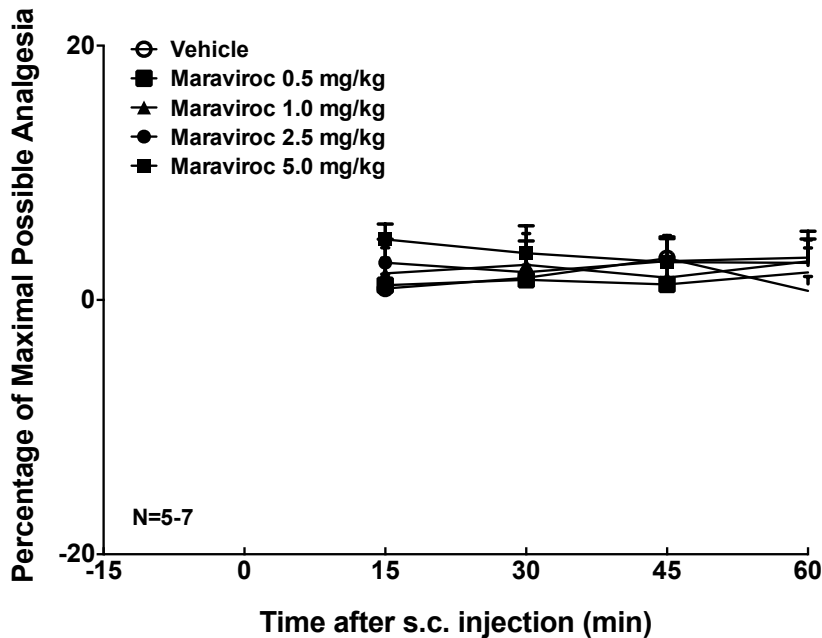


Figure 2. Antinociceptive effect of Maraviroc in the CWT. Rats (n=5-7/group) were injected s.c., into the dorsal surface of the body with vehicle (10% DMSO) or Maraviroc at time=0 min. Each animal was tested at 15, 30, 45 and 60 min after Maraviroc injection in the CWT. Maraviroc produced no significant antinociceptive effect ($p>0.05$, ANOVA test) in the CWT test.

Carrageenan test: Unrestrained rats were placed inside a clear plastic chamber with a glass floor that is part of the Hargreave's apparatus (IITC Life Science, Woodland Hills, CA, model 400). After a 5-minute period of habituation, the plantar surface of the hind paw was exposed to a beam of radiant heat (intensity =45) through the glass floor. The latency (secs) to paw withdrawal was automatically scored by the Plantar Test Apparatus (Model 400, IITC Life Science, Woodland Hills, CA) through the glass floor (Temperature=32°C). The animals were exposed to the heat beam 3 times over a 10-minute period before drug injection to establish the baseline pain value. Then, rats were injected s.c. into the plantar surface of the left hind paw with 0.1 ml of a 2% carrageenan solution, freshly prepared in normal saline. Latency to paw withdrawal was measured at 180 minutes post carrageenan injection based on preliminary data examining the effect of the irritant at different times. In addition, edema induced by carrageenan was also measured as described above. Measurements were taken immediately after the carrageenan injection and again at 180 minutes.

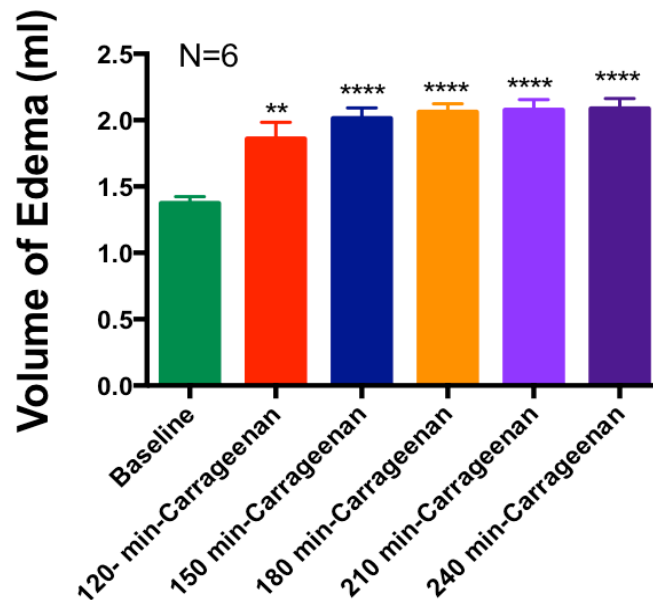


Figure. 3: Time course of carrageenan-induced edema. **p<0.01 or ****P<0.001 by ANOVA followed by Tukey's multiple comparisons test, compared to baseline.

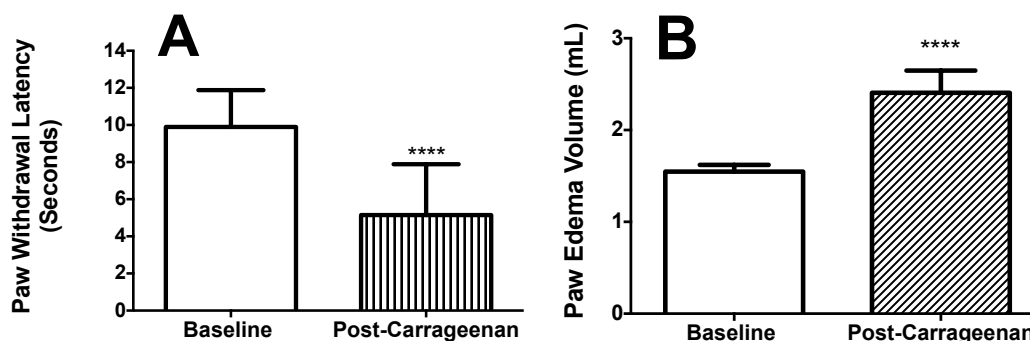


Figure. 4. Carrageenan-induced thermal sensitivity (2A) and edema (2B). Results are averaged values for 48 animals for each test, pooled from 8 different experiments. Data show a significant decrease in latency for paw withdrawal (panel A) and edema (panel B) at 180 minutes post carrageenan injection (* $p < 0.001$, unpaired t-test).

AMD3100, a chemokine receptor antagonist, active against the chemokine receptor, CXCR4, and blocking activity of CXCL12 (SDF-1) was tested in the carrageenan assay as per Specific Aim 1: Major Task 2: Subtask 1, to determine if, by itself, it had any inhibitory activity against the pain-inducing and inflammatory activity of the irritant (carrageenan). To carry out this test, rats were injected into the plantar surface of the left hind paw with carrageenan, as above for the baseline studies. At 195 minutes after administration of the carrageenan, AMD3100, in various doses dissolved in water, was injected s.c. in a volume of 0.1 ml into the dorsal surface of the body. The time of 195 minutes was chosen because, in subsequent experiments, animals will receive AMD3100 plus morphine. The morphine will be injected at 210 minutes and the AMD3100, 15 minutes earlier. Latencies to paw withdrawal after AMD3100 injection were tested at 240 minutes after the carrageenan injection. The percent of maximal possible antinociception (%MPA) for each animal was calculated using the following formula: $\%MPA = [(test\ latency - baseline\ latency) / (22 - baseline\ latency)] \times 100$. A cutoff limit of 22 seconds was set to avoid damage to the paw. To measure edema in AMD3100-treated animals, the paw was immersed in the conductive solution and fluid displacement was determined immediately after the radiant heat measure, at approximately 240 minutes. Edema data are the difference between the edema at 240 minutes minus 195 minutes. Vehicle control animals received a s.c. injection of 0.1 ml of water.

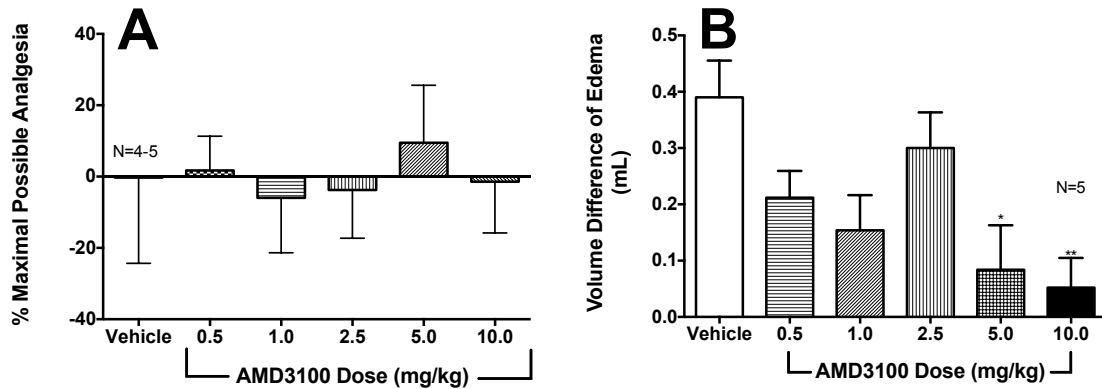


Figure 5. Dose-response effect of AMD3100 on pain and edema induced by carrageenan. All animals received carrageenan. Test groups received AMD3100 in doses of 0.5-10 mg/kg s.c. Vehicle group received water s.c. Panel A = latency to paw withdrawal, a measure of pain, in animals treated with carrageenan and various doses of AMD3100. No significant difference between any two groups ($p > 0.05$, (ANOVA followed by Tukey's multiple comparisons test). Panel B = edema in animals given carrageenan and various doses of AMD3100. (* $p < 0.05$, 5.0 mg/kg group; ** $p < 0.01$, 10.0 mg/kg group compared to vehicle (ANOVA followed by Tukey's multiple comparisons test).

Summary of carrageenan results: The results show that 1) carrageenan induces a reduced latency paw withdrawal in normal rats (indicating earlier onset of discomfort); 2) carrageenan induces marked paw swelling; 3) AMD3100 alone has no effect on paw withdrawal latency, a measure of pain; and 4) AMD3100 alone at 5.0 and 10.0 mg/kg significantly reduced paw edema.

Formalin Test: In this test, mice were injected s.c. into the plantar surface of the left hind paw with 20 μ l of a 2% formalin solution diluted in 0.9% saline. Animals were placed into a large glass jar where their actions could be monitored. Formalin injection causes intense licking of the injected paw, which prior studies in our laboratories have shown is maximal in intensity between 20 and 35 minutes. Licking was scored for each animal as the number of seconds licking occurred during that 15-minute period (20 to 35 minutes after injection). To determine if AMD3100 alone had an analgesic effect, various doses of this chemokine receptor antagonist, diluted in sterile water, were injected s.c., into the dorsal surface of the body, 30 minutes prior to the injection of formalin. Controls received water alone. Similarly, to determine if Maraviroc, another chemokine receptor antagonist, which binds to the chemokine receptor, CCR5, had analgesic activity when used alone, it was injected in separate experiments into mice challenged with formalin in the paw. Maraviroc was obtained from Sigma-Aldrich and dissolved in 5% DMSO.

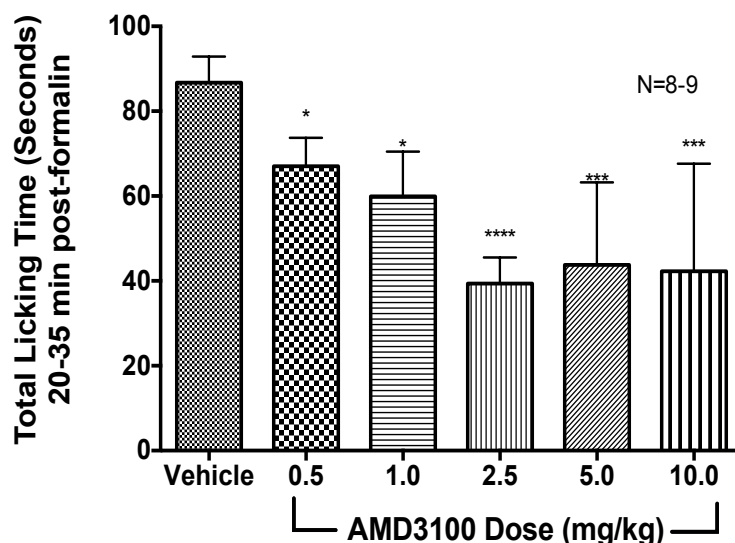


Figure 6. Effect of AMD3100 on licking response to formalin injection in the footpad of mice. Mice received AMD3100 (0.5-10 mg/kg) s.c. or water vehicle, 30 minutes prior to injection of a 2% formalin injection into the foot pad. All animals (n=8 to 9) received formalin. The number of seconds spent licking the injected paw was scored for 15 minutes beginning 20 minutes after the formalin injection. AMD3100 significantly reduced licking (* $p < 0.05$, ** $p < 0.01$ by unpaired t test, as compared to vehicle with formalin)

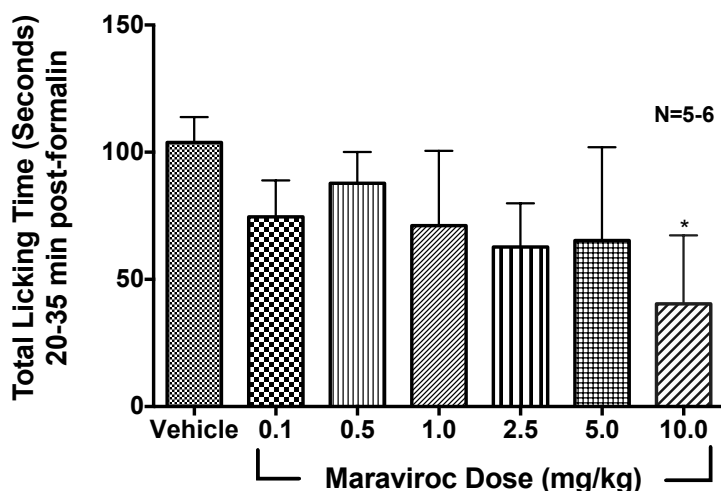


Figure 7. Effect of Maraviroc on licking response to formalin injection in the footpad of mice. Mice received Maraviroc (0.1-10 mg/kg) s.c., or 5% DMSO for vehicle controls, 30 minutes prior to injection of a 5% formalin injection into the foot pad. All animals (n=5 to 6) received formalin. The number of seconds spent licking the injected paw was scored for 15 minutes beginning 20 minutes after the formalin injection. Although Maraviroc showed evidence of analgesic activity at all doses, statistical significance (* $p < 0.05$, ANOVA followed by Tukey's multiple comparisons test) was achieved only at the highest dose tested.

Summary of formalin test results: In the formalin test, AMD3100 produced a dose-related reduction in paw licking, with statistically significant activity at doses between 0.5 and 10 mg/kg. The 2.5 mg/kg dose produced maximal activity, which was approximately a 50% reduction in licking. Maraviroc, in contrast, only reduced licking at a statistically significant level at the 10.0 mg/kg dose. The number of animals tested needs to be increased by repeating these experiments.

Summary of progress on Specific Aim 1: Major Task 2: Subtask 1: Test the chemokine receptor antagonists (CRAs), AMD3100 and Maraviroc, each separately, in three pain models: Cold water tail-flick test (CWT), carrageenan, and formalin.

Excellent progress has been made on this Aim, Task and Subtask. All 3 assays have been established and the effect of AMD3100 has been tested in each of them. Maraviroc has been tested in the CWT and the formalin test.

Specific Aim 2, Major task 1: Subtask 1: Establish the dose- and time-response curves for morphine in the formalin pain model.

In this test, mice were injected s.c. into the plantar surface of the left hind paw with 20 µl of a 5% formalin solution diluted in 0.9% saline. Animals were placed into a large glass jar where their actions could be monitored. Formalin injection causes intense licking of the injected paw that prior studies in our laboratories have shown is maximal in intensity between 20 and 35 minutes post-injection. Licking was scored for each animal as the number of seconds licking occurred during that 15-minute period (20 to 35 minutes after injection). To determine if morphine alone had an analgesic effect, various doses diluted in saline were injected s.c., into the dorsal surface of the body, 30 minutes prior to the injection of formalin. Controls received saline alone.

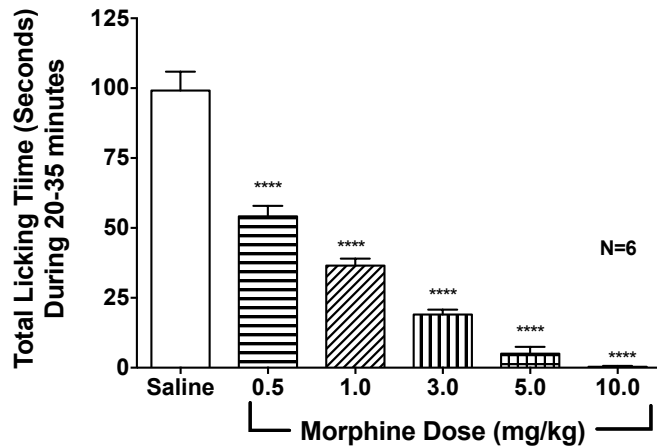


Figure 8: Effect of morphine on licking response to formalin injection in the footpad of mice. A decrease in licking time is a measure of antinociception. Mice received morphine (0.5-10 mg/kg) s.c., or saline vehicle, 30 minutes prior to a 20 μ l injection of a 5% formalin injection into the foot pad. All animals (n=6) received formalin. The number of seconds spent licking the injected paw was scored for 15 minutes beginning 20 minutes after the formalin injection. Morphine at all doses tested significantly reduced licking (**p<0.001 ANOVA followed by Tukey's multiple comparisons test). Morphine (0.5-10 mg/kg) or saline was injected s.c. The licking response measured 20-35 minutes after formalin injection showed a dose-related decrease in licking in response to all doses of morphine (p<0.001), compared to the saline group.

Subtask 2: Establish the dose- and time-response curves for morphine in the cold water tail-flick (CWT) pain model.

(See Specific Aim 1: Major Task 2: Subtask 1 for method for the CWT.

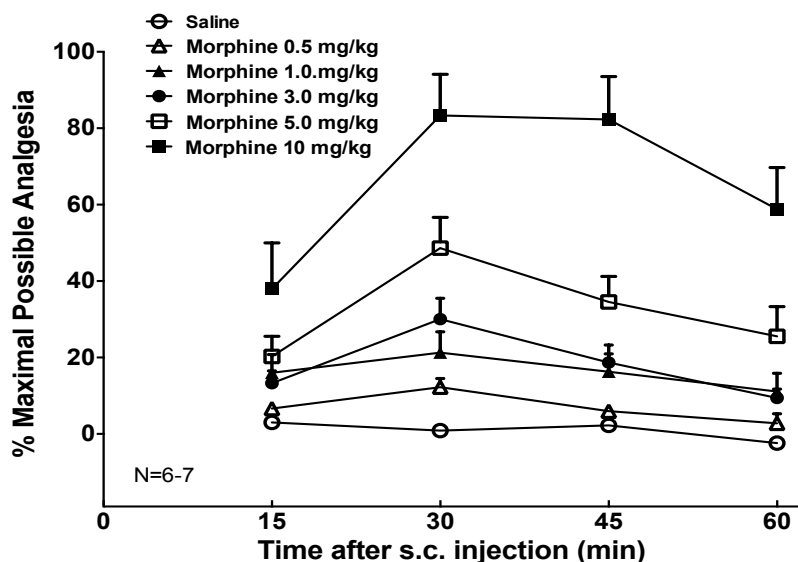


Figure 9. Effect of morphine dose and time of administration on antinociception in the cold water tail-flick test (CWT). Rats ($n=6-7/\text{group}$) were injected s.c., into the dorsal surface of the body with vehicle (saline) or morphine at time=0 min. Each animal was tested at 15, 30, 45 and 60 min after morphine injection in the CWT. Morphine resulted in a dose-related antinociceptive effect ($****p<0.0001$ by two-way ANOVA test) that was maximally evident between 30 and 45 minutes post opioid administration in the CWT test.

Subtask 3: Establish the dose- and time-response curves for morphine in the carrageenan pain model.

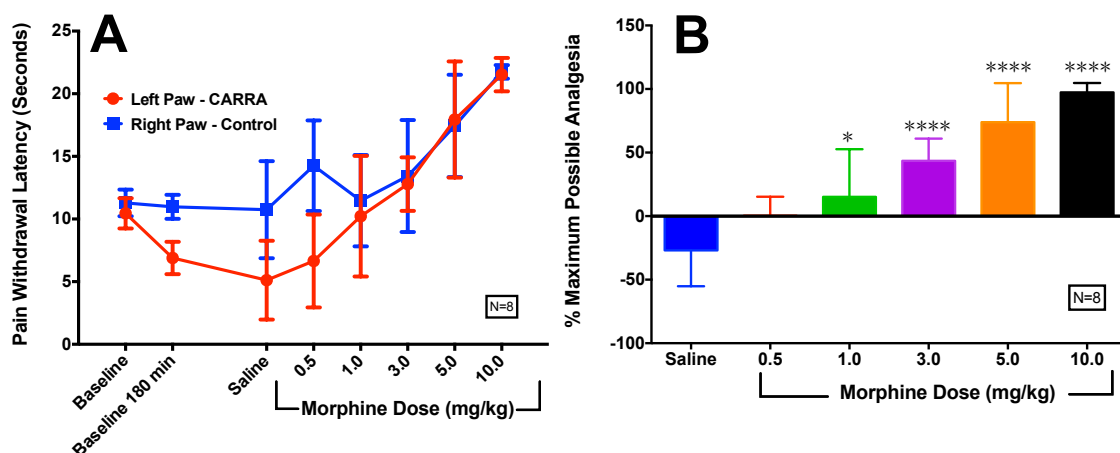


Figure 10: Dose-response effect of morphine on pain induced by carrageenan. All animals received carrageenan in the left rear paw and vehicle (saline) in the right rear paw. Test groups received morphine at various doses s.c. Vehicle group received saline s.c. Panel A: Comparison of effect of carrageenan and morphine on the left and right paws. Data are expressed as Pain Withdrawal Latency. ($*p<0.005$ by the two-way ANOVA test). Panel B: Effect of morphine on analgesia in the left paw injected with carrageenan, expressed as % Maximum Possible Analgesia. ($*p<0.05$, $****p<0.0001$, ANOVA following by Tukey's multiple comparisons test)

Figure 10 shows that carrageenan, by 180 minutes after injection into the left paw, induced a significant decrease in the time of paw withdrawal. A decrease in withdrawal time is an indication of sensitivity to pain. A similar decrease was not observed for the right paw, which received saline. Morphine caused a dose-dependent decrease in nociception over the range of 0.5 to 10 mg/kg. The analgesic effect of morphine was evident in both the left and right paws.

The effect of morphine in the carrageenan model was also examined with respect to reduction of edema.

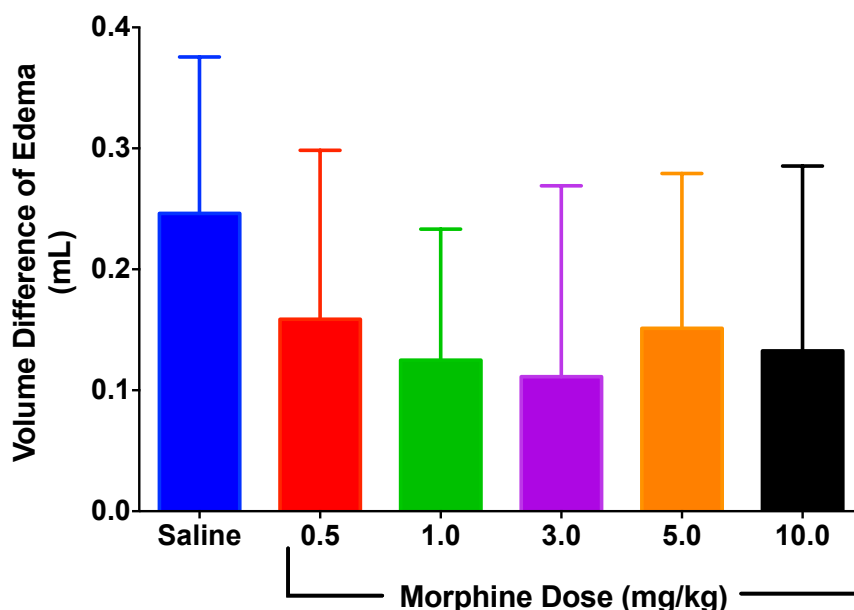


Figure 11. Dose-response effect of morphine on edema induced by 2% carrageenan. All animals received carrageenan in the left rear paw and vehicle (saline) in the right rear paw. At $t=+210$ minutes, test groups received morphine in various doses s.c. and vehicle group received saline s.c. Edema measurements after morphine injection were tested at 240 minutes after the carrageenan injection. Edema data are the difference between the edema at 240 minutes minus 210 minutes. No significant effect of morphine on edema in the carrageenan-injected paw was observed. ($p>0.5$, ANOVA followed by Tukey's multiple comparisons test)

Specific Aim 1: Major Task 3: Determine whether chemokine levels are altered in each of 3 pain models and by treatment with 2 chemokine receptor antagonists (CRAs)

Subtask 1 and Subtask 5: Analyze serum from animals injected with formalin or carrageenan or used in the cold water tail-flick test, with and without morphine, for levels of chemokines using the Luminex platform.

Methods overview: The Luminex system is a multiplex platform that allows simultaneous, quantitative determination of the levels of panels of chemokines and cytokines in as little as 25 μ l of serum. “Analyte” is the term used to describe the general class of chemokines and cytokines. These substances can also be called “mediators” in the immune response. The Luminex system can run multiple analytes at one time, but the price increases with the number of individual ones analyzed. We chose to use an 8-plex assay, which we felt gave a good range of molecules to be tested and was intermediate in price (approximately \$1,000/plate that can run 41 samples in duplicate). We chose to include some of the pro-inflammatory cytokines as well as chemokines in these assays. The assay works as follows. Customized kits are purchased from R&D Systems (Minneapolis, MN). The assay is carried out in a supplied 96-well plate. The sample to be analyzed is mixed with a supplied custom mix of magnetic beads bound with antibodies to the 8 analytes. Dilutions of standards of known concentrations of the 8 analytes and a zero value sample are also mixed with the bead cocktail. After incubations with supplied fluorescent reagents, the plates are read on a BioPlex 100/200 system instrument (BioRad, Richmond, CA). This system detects the fluorescence of the bound antibodies within each analyte group. The intensity of fluorescence is proportional to the amount of analyte present in the sample. The fluorescence produced by the test samples is obtained by calculation against the standard curves included in the assay run. These assays are sensitive in the picogram to nanogram range depending on the analyte. Thus, this type of assay gives highly quantitative results for the amounts of protein of a given analyte in a sample.

Preliminary experiments using serum from animals in the formalin test showed no measurable chemokines or cytokines in this Luminex. Serum samples were collected at the end of the pain assay, which is 35 minutes after formalin injection. For the Luminex assay we used a custom panel of analytes containing CXCL1/KC, IL-1 β , IL-6, CCL5/RANTES, CXCL2/MIP-2, CCL2/MCP-1, CXCL10/IP-10, and CXCL12/SDF-1 α . The serum showed no rise in any of these chemokines/cytokines, although the assay was working, as we obtained excellent standard curves with the kits. We hypothesized that the lack of reactivity in serum might be due to the time of serum harvest, and carried out another preliminary experiment in which serum was collected at 35, 90, and 180 minutes after the formalin injection. Extending the time did not reveal greater levels of chemokines/cytokines. We also tried to extract the paw tissue where the formalin was injected to examine the tissue for levels of chemokines/cytokines. We followed published methods for extracting the tissue with protease inhibitors to prevent degradation of the chemokines/cytokines. This involved mechanical homogenization of the tissue in phosphate-buffered saline (PBS) with the addition of Halt[®] Protease Inhibitor (Pierce Chemical Co., Rockford, IL), followed by high-speed microcentrifugation and collection of the supernatant. Again, the results were negative. As formalin is injected into the footpad, it was decided to use an extract of the draining popliteal lymph node for analysis of immune mediators, instead of serum. The lymph node was first mechanically

homogenized in PBS, then an equal volume of Cell Lysis Buffer 2 (R&D Systems) was added, followed by a 30-minute incubation at room temperature with gentle agitation. The samples were then centrifuged at 12,000 rpm at 4° C for 10 min. Supernatants were collected and stored at -80° C until assay. Using this method we did find measurable levels of several of the chemokines and cytokines. Based on these preliminary studies it was decided to analyze the popliteal lymph node, rather than serum as specified in the SOW for subtasks 1 and 5. However, It was decided to continue to harvest serum, the popliteal lymph node, the spleen, and paw tissue (from the formalin and carrageenan injections) from each animal used in the pain studies in case we needed them for further analysis. The serum and tissue were frozen at -80° C and can be defrosted and extracted as needed.

Results:

Formalin alone and formalin plus AMD3100: The data shown in figure 12 were obtained from animals that were injected with formalin alone or with escalating doses of AMD3100. The pain (licking response) and edema measurements on these mice are shown in Figures 6 and 7 of this report. In Figs 6 and 7, the bar marked “vehicle” corresponds to “formalin alone”. The other bars represent animals that received formalin plus AMD3100 (0.5-10 mg/kg). In Fig.12, “background” represents values for animals that received “no treatment and “formalin” represents values of animals that received formalin + a s.c. injection of water, which is the vehicle used to dissolve the AMD3100.

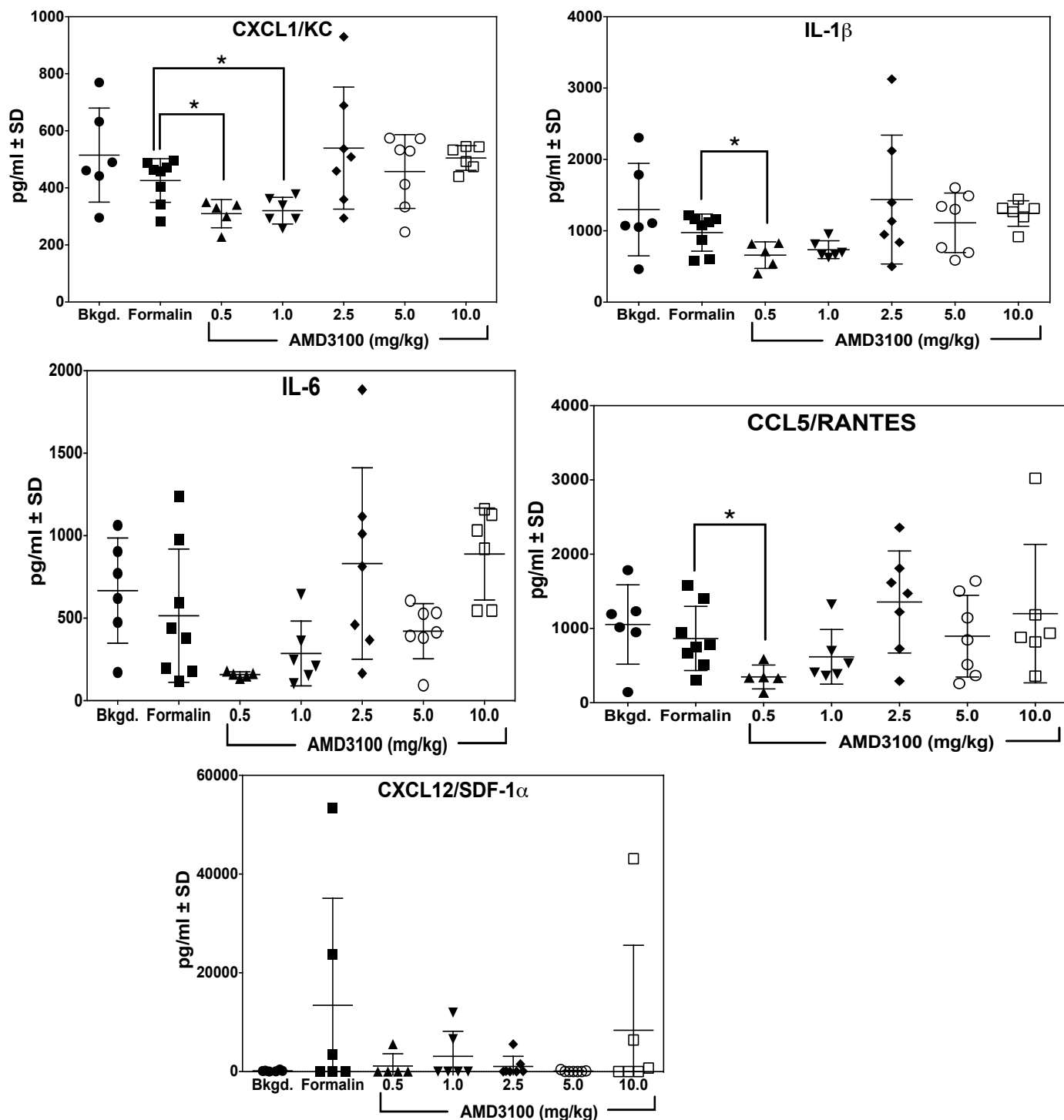


Figure. 12: Effect of formalin alone or in combination with various doses of AMD3100, on levels of selected chemokines and cytokines in the popliteal lymph node of mice. Levels of 8 analytes were measured in extracts of the lymph node draining the formalin-injected paw of each mouse ($n=5$ to 8). There were no detectable levels of CXCL2/MIP-2; CCL2/MCP-1 or IP-10. There were detectable levels of the other 5 analytes, which are shown as dots, representing the value for each individual mouse for each analyte and each treatment. (* $p < 0.05$)

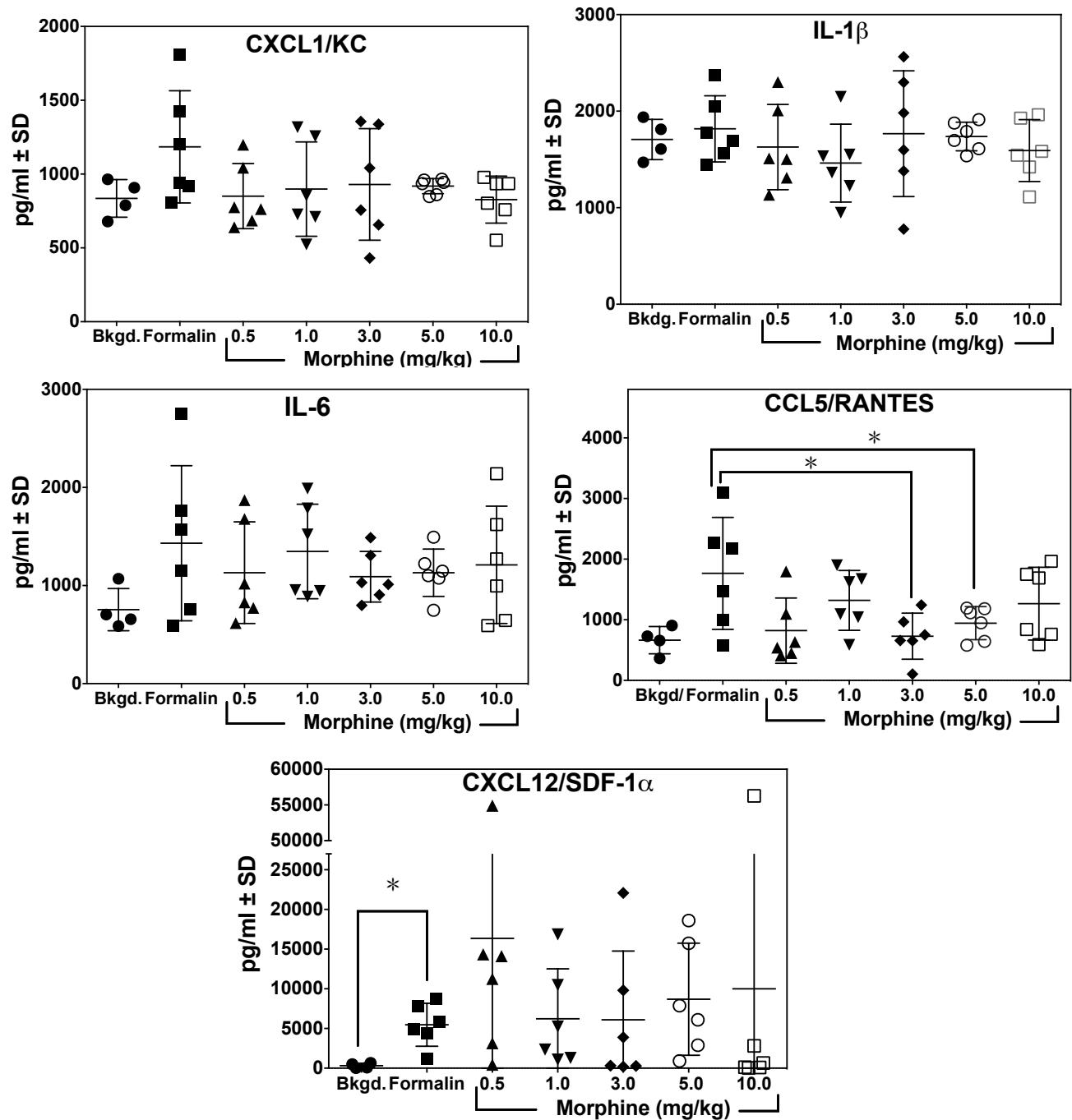


Figure 13: Effect of formalin, alone or in combination with various doses of morphine, on levels of selected chemokines and cytokines in the popliteal lymph node of mice. Levels of 8 analytes were measured in extracts of the lymph node draining the formalin-injected paw of each mouse (n=5-8). There were no detectable levels of CXCL2/MIP-2; CCL2/MCP-1 or CXCL10/IP-10. There were detectable levels of the other 5 analytes, which are shown as dots, representing the value for each individual mouse for each analyte and each treatment.

* $p < 0.05$

Examination of the data shows that formalin did not significantly elevate the levels of CCL5, CXCL12, CXCL1, IL-6 or IL-1 β above background. However, it appears that AMD3100, at the lowest dose (0.5 mg/kg), reduced the levels of CXCL1, CCL5, and IL-1 β . These results do not show a strong correlation with the pain data, because while AMD3100 diminished pain at the 0.5 mg/kg dose, more robust results against pain were observed at AMD3100 doses of 2.5 to 10.0 mg/kg.

Formalin alone and formalin plus morphine: Figure 13 presents the results of giving mice formalin alone or formalin plus morphine. These samples were taken from mice whose pain responses are shown in Figure 8. Morphine, in doses from 0.5 mg/kg to 10 mg/kg, had no significant effect on the formalin-induced levels of these cytokines, and there was no clear dose-related effect.

Compilation of data on formalin induction of chemokines and cytokines:

Examination of the data in figures 12 and 13 on levels of chemokines and cytokines detected in the mouse popliteal lymph node after formalin alone shows only a weak inflammatory response provoked by this irritant. In Figure 13, the formalin injection did not significantly raise the levels CXCL1, CCL5, CXCL12, IL-1 or IL-6.

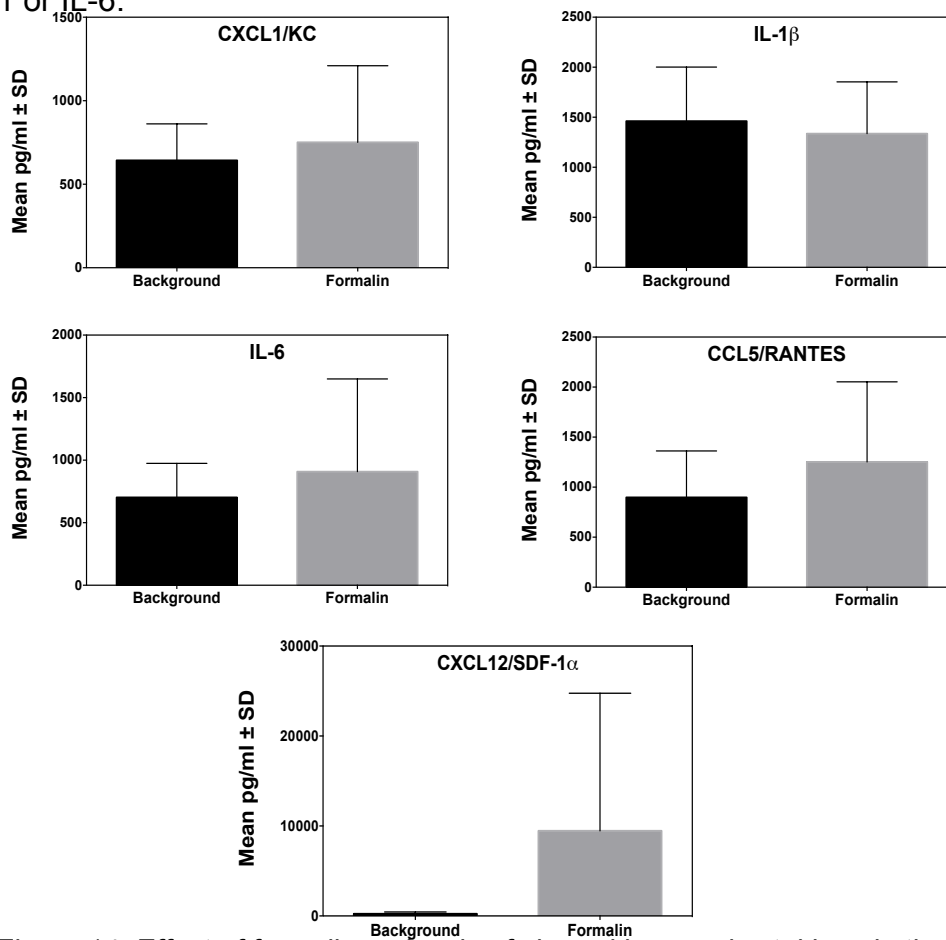


Figure 14. Effect of formalin on levels of chemokines and cytokines in the draining popliteal lymph node of mice injected with formalin into the left hind footpad. n=14, $p>0.5$

To better assess the ability of formalin to induce inflammation, the data on “background” and “formalin” alone in these two figures were combined (Figure 14) for the five mediators where there were measurable results. With an “n” of 14, it is clear that there was no significant effect of the formalin on production of these analytes.

Carrageenan alone and carrageenan plus morphine: A Luminex assay was carried out on rats that had received carrageenan into the plantar surface of the left hind paw. The draining popliteal lymph node, as well as the draining node from the contralateral paw, were tested for levels of chemokines and cytokines. There is a much more limited number of analytes available for the rat, as compared to the mouse. The panel used in this experiment consisted of IL-1 β IL-18, TNF- α , and CXCL2/CINC-3.

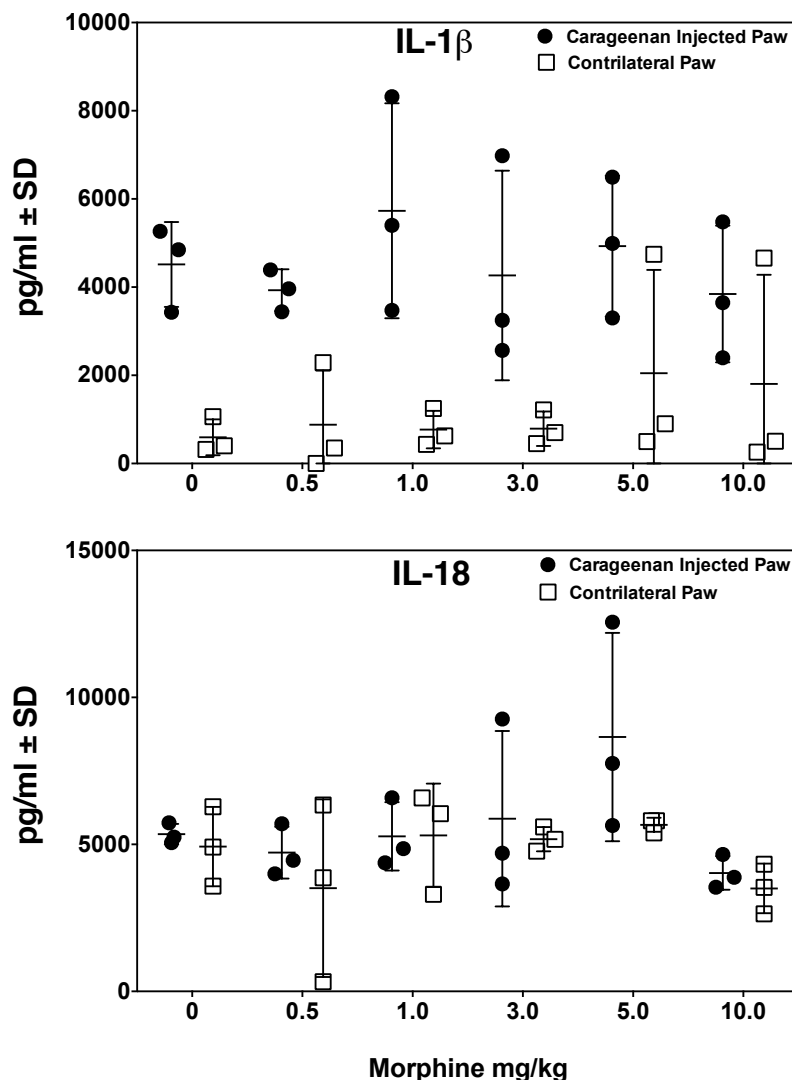


Figure 15: Effect of carrageenan alone or with morphine on levels of chemokines and cytokines. Rats received carrageenan into the left rear paw and saline into the contralateral right paw (n=3). The rats were tested for pain responses to radiant heat (see explanation of the carrageenan test that precedes Fig. 3), and

the draining popliteal lymph nodes of each paw were harvested at the end of the pain assay and analyzed for levels of CXCL2, TNF- α , IL-1 β and IL-18. Only IL-1 β and IL-18 gave measureable levels of cytokines.

Results: In a preliminary experiment analyzing the results of 3 animals, morphine, at doses between 0.5 to 10 mg/kg, did not affect the levels of either IL-1 β or IL-18 in the paw receiving carrageenan or in the contralateral control paw.

Subtask 4: Analyze paw or draining lymph node tissue from animals used in all 3 pain tests for chemokine proteins using the Proteome Profiler Array (R&D Systems)

It was decided to move to Subtask 4 and use the Proteome Profiler Array (PPA) to be able to survey a larger panel of chemokines and cytokines. The PPA kit has 4 identical membranes in which are embedded antibodies, as individual spots, to a panel of 29 analytes. The samples are loaded onto the membrane and bind to the antibodies with the proper specificity. The binding is analyzed by incubation of the membrane with horseradish peroxidase-labeled detection antibodies to the individual analytes. After incubation and washing, the membranes are developed by incubation with the supplied chemiluminescence reagent. The luminescence is detected by digital photography with a Fujifilm LAS-100 Digital Imaging system, and analyzed by Image Gauge[®] software (Fuji Ltd., Tokyo, Japan). This type of assay is a screen for levels of protein and is only semi-quantitative. Results are expressed as Arbitrary Units (AU)/mg protein. The intensity of the luminescence is positively correlated with the amount of protein bound. The samples chosen were from a preliminary experiment (n=3) in which rats were injected with carrageenan, alone or with morphine (data not shown) The kit comes with only 4 membranes so equal amounts of sample from 5 animals in each of 4 treatment groups were pooled and their protein content determined using a BCA Protein Assay (Pierce Chemical Co.). Pooled samples used for binding contained approximately 1 mg/ml protein. The groups chosen were carrageenan paw lymph node/saline s.c., contralateral paw, no carrageenan/saline s.c., carrageenan paw/morphine s.c., and contralateral paw, no carrageenan/morphine. Only one dose of morphine could be analyzed in a single array, and the 10.0 mg/kg was chosen.

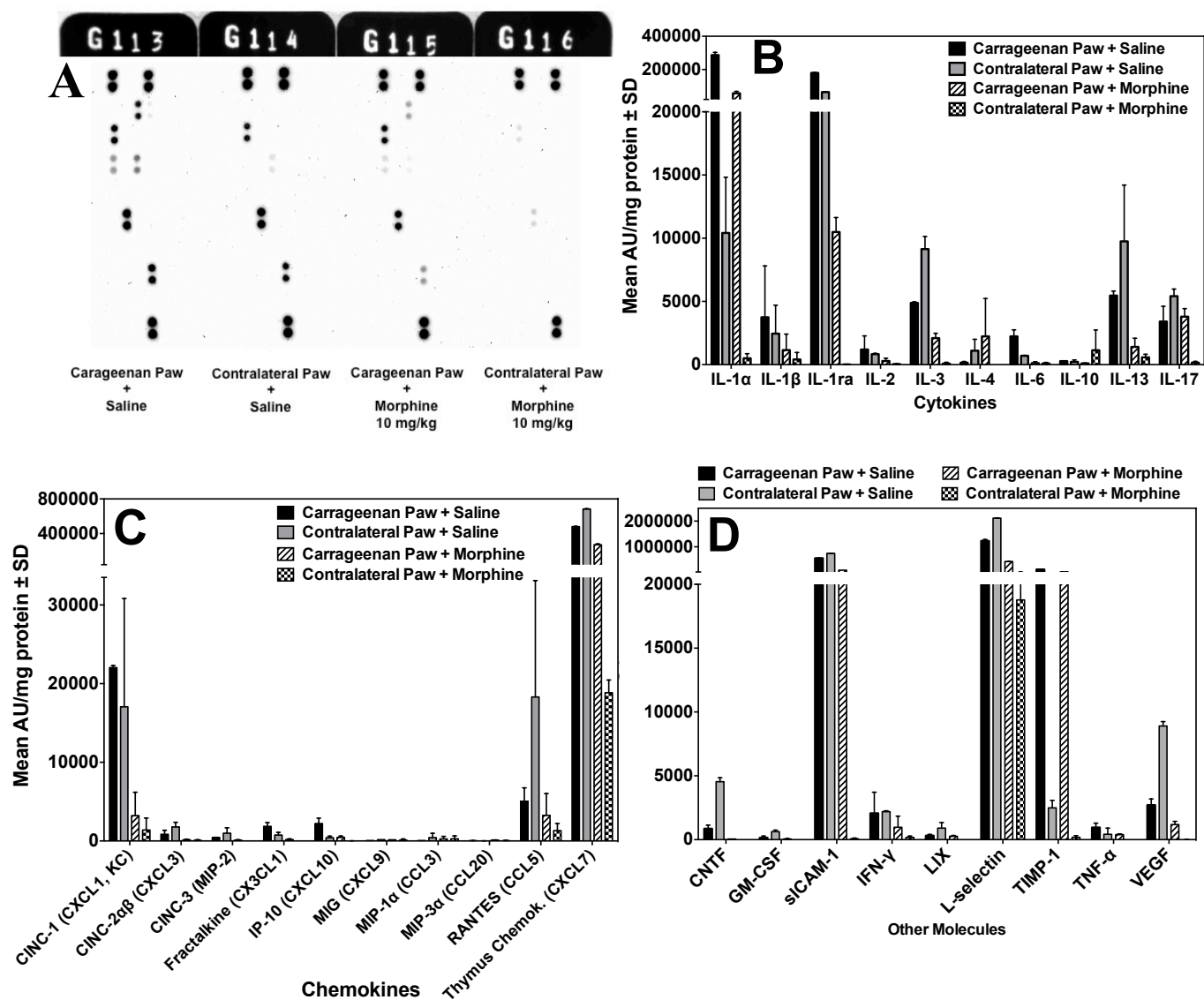


Figure 16: Chemokine and cytokine levels in the popliteal lymph nodes of rats injected into the footpad with carrageenan, with or without morphine. Each sample is a pool of popliteal lymph node extractions of 5 rats. Panel A: Visualization of spots developed in the array. Panels B-D: Results were quantitated using Image Gauge[®] software with a Fuji Digital Imaging System to measure intensity of the luminescence of spots. B=chemokines; C =cytokines, and D=other molecules.

Results: The results shown in the bar graphs compare the levels of various chemokines, cytokines and adhesion factors in rats receiving carrageenan, an inflammatory stimulus, with or without morphine. Comparison of the bars for carrageenan paw + saline with contralateral paw + saline indicates the degree of the immune response induced by the carrageenan injection. Carrageenan gave significant ($p < 0.005$) elevation of IL-1 α and TIMP-1. Several chemokines/cytokines and adhesion molecules were elevated in both the carrageenan-injected paw and the contralateral control paw including IL-1 α , CXCL1, CXCL7, sICAM-1, and L-selectin. Morphine significantly depressed levels of IL-1 α and TIMP-1 in both the carrageenan and contralateral paws ($p < 0.005$). It also depressed levels of IL-1 α , CXCL7, sICAM-1, and L-selectin most dramatically in the contralateral paw. The significance of the changes in these particular immune molecules is to be determined. This type of array analysis appears to be a more optimal way to analyze the changes in immune mediators, with confirmation of the results using Luminex assays where reagents are available.

Future Plans

Specific Aim 1: Major Task 2: Subtask 1.

Test the chemokine receptor antagonists AMD3100 and Maraviroc, each separately in three pain models: cold water, carrageenan and formalin.

Overview: Most of the tasks in this aim have been accomplished. The following will be carried out:

1. Repeat AMD3100 in the carrageenan test to increase the "n".
2. Repeat Maraviroc in the formalin test to increase the "n".
3. Test the effect of Maraviroc in the carrageenan test

Specific Aim 1: Major Task 3: Subtasks 2, 3, 4, 6 and 7.

Experiments to carry out the Proteome Profiler Array and the RT2 Profiler PCR array are currently in the process of being carried out on animals receiving morphine and/or CRAs in the formalin paw injection, carrageenan paw injection, and cold water tail flick pain assays.

Specific Aim 2: Major Task 1: Subtask 4.

Start experiments on the incisional pain model and establish dose- and time-response curves for morphine.

Specific Aim 2: Major Task 2.

Using the formalin, the cold water, and the carrageenan pain tests, carry out studies using morphine plus two chemokine receptor inhibitors (antagonists), AMD3100 and Maraviroc, tested individually.

Specific Aim 2: Major Task 3.

Using the formalin, the cold water, and the carrageenan pain tests, start to carry out studies using morphine plus two additional chemokine receptor inhibitors (antagonists) for CCR2 and CX3CR1, tested individually.

Specific Aim 2: Major Task 5.

Carry out studies identified above under Major Tasks 1 to 4 substituting Oxycontin for morphine in selected assays.

Specific Aim 2: Major Task 6.

Measure levels of chemokines in tasks 2 to 5 (as part of Aim 2 above) using the same assays specified under subtasks of Aim 1, Major Task 3. As noted in the current Progress Report, analysis of serum may not be optimal for measuring levels of chemokines. Instead, the draining popliteal lymph node or splenic tissue may be tested for chemokine levels. For each pain model, the Proteome Array and/or the RT₂ Profiler Array will be used to help identify the chemokines and cytokines that are altered. These data will be verified and quantitated using the Luminex and/or ELISA assays.

Specific Aim 3:

Evaluate whether various combinations of the chemokine receptor antagonists (CRA) are more efficacious than the use of a single CRA in conjunction with the analgesic agent.

Major Task 1: Subtask 1. Measure effects of the CRA combinations on pain.

Major Task 1: Subtask 2. Determine the effects of the CRA combinations on chemokine levels in serum and tissues.

Major Task 1: Subtask 3. Compare Oxycontin with morphine in selective cases for pain where a combination AMD3100 and Maraviroc is used.

Major Task 1: Subtask 4. Compare Oxycontin and morphine in selective cases used in Subtask 3 for chemokine levels.

4. KEY RESEARCH ACCOMPLISHMENTS: This grant was awarded on July 15, 2015. It took almost 6 months to get the required IACUC/ACURO documents approved, and to hire and train all of the personnel. In the remaining 6 months we made excellent progress towards fulfilling the Aims and Tasks of the proposal. We established baselines for the 3 pain models that were to be tested in year 1: the cold water tail-flick test, the carrageenan pain model, and the formalin pain model. Morphine dose-response curves were obtained in each model. The effect of one of the chemokine receptor antagonists (AMD3100) was measured in all 3 pain models. The second chemokine receptor to be tested in year 1, Maraviroc, was evaluated in 2 of the 3 pain models. In the SOW, the evaluation of these 2 CRAs was projected to be completed between months 3 and 15, so there are still 3 months left to complete this task. For some of the assays we anticipate repeating experiments to increase the “n”, and to confirm results. Also, we had proposed starting the experiments of the combinations of

the chemokine receptor antagonists and morphine in the 3 pain models. This aspect of the work has not started but is almost ready to begin. We have firmly established the conditions needed for the Luminex multiplex assay to assess panels of chemokines and cytokines in serum and tissue extracts (spleen, popliteal lymph node, paw tissue) and have measured levels of chemokines and pro-inflammatory cytokines in mice receiving formalin plus AMD3100 and rats receiving carrageenan plus morphine. We have also carried out one experiment using the Proteome Profile Array on popliteal lymph nodes from animals receiving carrageen alone or with morphine. We are about to embark on experiments testing mRNA levels of chemokines and cytokines using the RT₂ Profiler PCR Array kit. We had projected in the SOW that some of these experiments would take until month 14 to complete, so we are close to the projected time frame, in spite of the 6-month, rather than the anticipated 3-month, delayed start of the project.

5. CONCLUSIONS:

We completed the first year of this 4-year grant. Due to the six-month delay in obtaining IACUC and ACURO approval and hiring personnel, laboratory work was not begun until January, 2016. A large proportion of the experiments planned for the first year have been completed in the last 6 months and steady progress has been made on the remainder, it has not been possible to complete all of the experiments proposed for year 1. Those will be completed first, as we move to the proposed year 2 experiments. We will prioritize the work essentially by following the order of the Aims, so that Aim 1 will be highest on the list followed by experiments in Aims 2 and then 3.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Nothing to report.

7. INVENTIONS, PATENTS AND LICENSES:

Nothing to report.

8. REPORTABLE OUTCOMES:

Nothing to report.

9. OTHER ACHIEVEMENTS:

Nothing to report.

10. PERSONNEL

Name: Martin W. Adler, Ph.D.
Project Role: PI
Nearest person months worked: 5
Contribution to Project: Overall direction of this project, including involvement in planning and design of all phases of the research, as well as choice of drugs and doses.

Name: Toby K. Eisenstein, Ph.D.
Project Role: Co-Investigator
Nearest person months worked: 2
Contribution to Project: Responsible for all experiments related to measurements of chemokines after induction of pain in the various models, including following the use of chemokine receptor antagonists and drugs.

Name: Alan Cowan, Ph.D.
Project Role: Co-Investigator
Nearest person months worked: 3
Contribution to Project: Prime responsibility for oversight of all experiments involving testing of the drugs in pain models and in evaluation of the results.

Name: Scott M. Rawls, Ph.D.
Project Role: Co-Investigator
Nearest person months worked: 2
Contribution to Project: Responsible for oversight of experimental design using chemokine receptor antagonists (CRA), choice of specific CRAs, and oversight of experiments involving CRAs.

Name: Ronald J. Tallarida, Ph.D.
Project Role: Co-Investigator
Nearest person months worked: 2
Contribution to Project: Responsible for design and interpretation of dose-response, time-response, and multiple drug studies.

Name: Xiaohong Chen, Ph.D.
Project Role: Associate Scientist
Nearest person months worked: 12
Contribution to Project: In charge of conducting experiments with animals and preparing test solutions; also graphs results.

Name: Ellen B. Geller
Project Role: Scientific Coordinator
Nearest person months worked: 3
Contribution to project: Assists in coordinating the work from all of the laboratories involved in this study, and also keeps the inventory for and dispenses any controlled substances used in this project.

Name: Joseph Meissler
Project Role: Laboratory Manager
Nearest person months worked: 3
Contribution to project: Responsible for measurement of chemokines after the various treatments.

Name: Jason Dicola
Project Role: Research Assistant
Nearest person months worked: 6
Contribution to project: Assists Xiaohong Chen in conducting experiments and also conducts literature searches.

Name: Mia N. Watson
Project Role: Laboratory Assistant
Nearest person months worked: 5
Contribution to project: Assists Xiaohong Chen in conducting experiments and collecting data.

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